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Does Temperature Affect Diploid Male Production in *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae)?

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Abstract.—As in many other hymenopterans, sex in *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) is determined by single-locus complementary sex determination. Thus, unfertilized eggs become haploid males and fertilized eggs that are homozygous and heterozygous at the sex locus develop into diploid males and females, respectively. We investigated the effect of temperature during development on the production and survivorship of diploid males. Females were allowed to oviposit at 20°C and 27°C and progeny remained at the same temperature throughout development. Diploid males were produced at both temperature regimes indicating that temperature does not affect sex determination in *H. hebetor*. However, temperature did affect diploid male survivorship, which was higher at the low temperature.

In haplo-diploid hymenopterans, sex is usually determined at oviposition with fertilized eggs developing as females and unfertilized eggs developing as males (Cook 2002). Haplo-diploidy is achieved in many hymenopterans by a mode of sex determination known as single-locus complementary sex determination (Whiting 1943, Cook 1993, Cook & Crozier 1995, Butcher et al. 2000a, b, Beukeboom 2001). In this system, sex is determined at a single genetic locus with multiple alleles. Sex-locus heterozygotes develop as females and sex-locus homozygotes develop as diploid males. Haploid males (hemizygotes) are produced from unfertilized eggs as in standard haplo-diploidy. Diploid males are inviable or sterile and females that mate with diploid males produce all male (haploid) offspring or, rarely, sterile triploid females (Bostian 1936, Stouthamer et al. 1992, Cook & Crozier 1995, Holloway et al. 1999).

As it is currently understood, complementary sex determination (CSD) is a form of genotypic sex determination in which sex is determined at fertilization and does not change over the course of the organism's life. Genotypic sex determination can be contrasted with sex determination mediated by cytoplasmic factors (Stouthamer et al. 2002) and with environmental sex determination, in which sex is determined by environmental factors such as temperature (Cook 2002). Temperature-dependent sex determination is quite common in reptiles and some other vertebrates (Bull 1983) and there is evidence for a mixture of genotypic and temperature-dependent sex determination in some of these species (Kraak & Pen 2002).

Investigations of temperature-dependent sex determination in insects are rare, but an early study on CSD in the parasitoid *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) reported a decreased incidence of diploid males in the offspring of females that were held and allowed to oviposit at 20°C rather than at 30°C (Whiting & Anderson 1932). Whiting and An-

derson's study suggested the possibility of an important link between temperature and sex determination (i.e., sex-allele homozygotes developing as females at low temperatures), but their results could also have been explained by decreased survivorship of diploid males at the lower temperature. Also, the time period during which sex determination would have occurred was poorly defined in their study since females and their offspring were held at the different temperatures for several days prior to, and following oviposition. Three more recent studies have also suggested that sex determination may be dependent upon temperature in parasitoids, and in all of these cases the critical sex determination stage was identified as the egg or early larval stage. Butcher et al. (1996, 1998) reported that *Diadegma chrysostictos* (Gmelin) (Hymenoptera: Ichneumonidae) and the sexual strain of *Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae) produced diploid males only when reared from the egg or early larval stage at temperatures exceeding 22°C, although Beukeboom (2001) could not repeat these results with *V. canescens*. An additional study reported that *H. hebetor* females allowed to oviposit at low temperature produced few or no diploid males despite intense inbreeding (Butcher 1998, personal communication). This result is consistent with the earlier findings of Whiting and Anderson (1932) but the critical time for sex determination was identified as being the egg or early larva. Here, we revisit Butcher's findings on temperature-dependent sex determination in *H. hebetor* by investigating the effects of developmental temperature on diploid male production and survival.

MATERIALS AND METHODS

Background on *H. hebetor*

Habrobracon hebetor is a gregarious ectoparasitoid of several species of phycitine pyralid moths (Heimpel et al. 1997). Females inject paralyzing venom into their hosts and lay three to twenty eggs on the surface of the host (Benson 1973). In the laboratory, *H. hebetor* females usually fertilize about two thirds of their eggs resulting in the production of a female-biased secondary sex ratio by outcrossed females (Petters & Mettus 1980, Antolin & Strand 1992, Heimpel et al. 1997, Ode et al. 1997). However, under CSD, the secondary sex ratio is altered when males and females share a sex allele since half of the fertilized eggs develop into diploid males. In *H. hebetor*, between 90 and 95% of diploid males typically die in the egg stage (Heimpel et al. 1999). Thus, females mated to males sharing a sex allele produce broods that are reduced in size by approximately one third and that have an even or slightly male-biased secondary sex ratio (Petters & Mettus 1980, Heimpel et al. 1999). Extreme polymorphism at the sex locus (Whiting 1943, Heimpel et al. 1999) and outcrossing (Antolin & Strand 1992, Ode et al. 1995) make the production of diploid males rare in the field (Antolin et al. 2003). However, conditions of inbreeding and restricted genetic diversity can lead to the production of diploid males (Whiting 1943, Heimpel et al. 1999).

Experimental *H. hebetor* were obtained from a colony reared on *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) larvae in an environmental chamber at the University of Minnesota at 25° C, 75% RH, and 16:8 photocycle (L:D). Two strains of *H. hebetor* were used. One strain carried a recessive eye color mutant exhibiting pale green eyes. This strain dates back to early work on *H. hebetor* done by P.W. Whiting and associates (see Whiting 1961) and retains the label "Oi". The second strain is a wild-type (black eye) strain collected in Kobe, Japan. Both strains were kept as separate 2-allele isolines initiated by mother-son matings and continued by brother-sister matings (see Heimpel et al. 1999). To avoid any confounding effects of *Wolbachia*, which is commonly present in

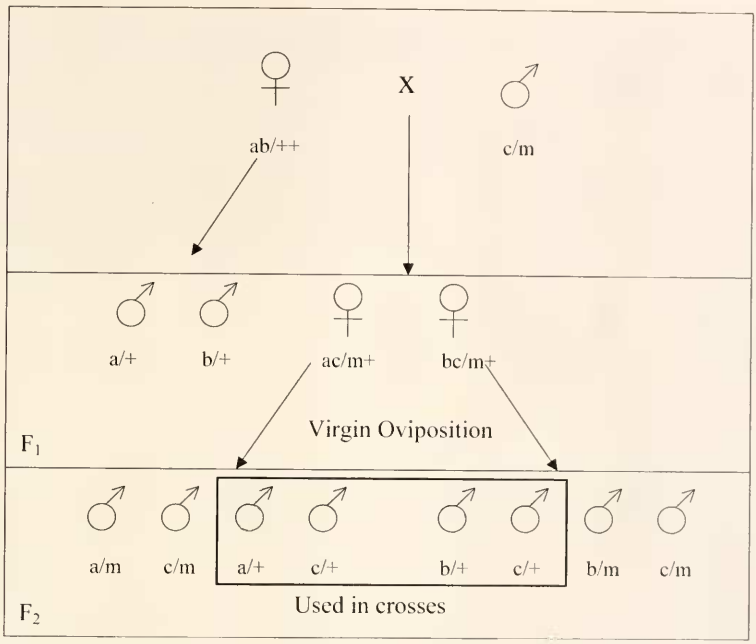

Fig. 1. Crosses showing production of recombinant males. Parents are from two unrelated isolines and presumed not to share any sex alleles. Sex alleles (a–c) and eye color (+ = wild type, black; m = mutant type, green) are indicated below each individual. The allele 'c' is used as a designator for one of two alleles present in the mutant line that differ from both of the alleles in the wild-type line. Virgin oviposition of F_1 females resulted in only recombinant male progeny, half of which were black-eyed. Black-eyed males were then mated with mutant females from the original parental mutant isoline, which carry the 'c' sex allele.

H. hebetor (M.F.A., L.A.W. & G.E.H. unpublished), we used the progeny from a cured isoline that were fed 10mg/ml rifampicin in 10% honey for 72 hours. The absence of *Wolbachia* was confirmed before and after the experiment by using a primer pair for a section of the *Wolbachia* wsp gene (Zhou et al. 1998). In these amplifications, uncured *H. hebetor* used as controls exhibited the expected 590 to 632-bp product (Zhou et al. 1998), and the offspring of cured *H. hebetor* showed no product. *Plodia interpunctella* were reared on a diet of wheat bran, chick feed, corn meal, glycerol, honey, and water (ca 43:30:14:9:2:2 by volume) at 25° C, 75% RH, and a 16:8 photocycle.

Experimental Crosses

Crosses were designed to ensure matings among males and females sharing a sex allele (henceforth referred to as 'matched' matings) and eye color markers were used to distinguish diploid from haploid males. First, recombinant *H. hebetor* males were produced from matings between wild type and mutant adults (Fig. 1). These recombinant males were then mated with virgin females from the maternal mutant isoline. One-half of the fertilized offspring of these shared-allele matings will be diploid males, recognizable by paternal inheritance of the wild-type eye color (black). Haploid males, which develop from unfertilized eggs, retain the maternal mutant eye color (green).

Each of twenty mated females were given two *P. interpunctella* for oviposition and a drop of honey each day until their death. Females were allowed to oviposit at 20° C for 2 consecutive days and then transferred to 27° C to oviposit for one day before returning to 20° C. We gave *H. hebetor* two days to oviposit at the cool tempera-

Table 1. Means (± S.E.M.) of the numbers of eggs laid per *H. hebetor* female, the egg hatch rate, the overall developmental mortality, the proportion of adult offspring that were females, and estimates of the fertilization rate and diploid male survivorship.

	Matched matings (n = 10)		Unmatched matings (n = 10)	
	20°C	27°C	20°C	27°C
Eggs laid/female	134.6 ± 6.14	134 ± 8.98	127.3 ± 9.26	104.7 ± 14.66
Egg hatch rate	0.78 ± 0.02	0.76 ± 0.02	0.91 ± 0.02	0.88 ± 0.02
Dev. Mortality	0.39 ± 0.03	0.38 ± 0.04	0.15 ± 0.03	0.14 ± 0.03
Secondary sex ratio (Proportion female)	0.39 ± 0.05	0.38 ± 0.05	0.60 ± 0.03	0.58 ± 0.06
Fertilization rate	0.57 ± 0.06	0.55 ± 0.06	0.60 ± 0.03	0.58 ± 0.06
Diploid male survival	0.6 ± 0.01	0.02 ± 0.01	N/A	N/A

ture because the oviposition rate is lower at 20° C than at 27° C. When females were transferred to another temperature, hosts were not provided until females had acclimatized to the new temperature (ca 2 hours). Eggs produced by mothers were transferred daily to freshly paralyzed, egg-free *P. interpunctella* using a blunt probe. Eggs and *P. interpunctella* were kept in 35 × 10 mm plastic petri dishes (Sarstedt Series #83.1800). Three eggs were placed on the ventral side of each paralyzed *P. interpunctella*, one near the head, one between the first and second pairs of prolegs, and one near the posterior, and were checked daily for hatching. Egg to adult development took place in an environmental chamber at 20° or 27° C, 75% RH, and a 16:8 L:D photocycle.

Data Analysis

Data recorded included the number of eggs laid and the egg hatch rate, larval—adult developmental success, and the sex and eye color of eclosing adults. Each mating was classified as "matched" if any diploid males were produced, and "unmatched" if no diploid males were produced. All black-eyed males were identified as diploids. Fertilization rate was estimated differently for matched ((total females × 2)/((total females × 2) + total haploid males)) and unmatched matings (number of females/total adult progeny). Diploid male survivorship was estimated as (diploid males/females) on a per-family basis. Data were analyzed using matched pairs analysis with each female serving as its own control (SAS Institute 1995). Two females produced all male progeny and were excluded from analyses as it was assumed that they had not mated.

RESULTS

Temperature during development did not affect production of diploid males by *H. hebetor*. We found evidence for single-locus CSD at both 20° C and 27° C, with patterns for both temperatures very similar to previous studies (Whiting 1943, Petters & Mettus 1980, Heimpel et al. 1999). Table 1 summarizes the mean ± SEM egg hatch rate, overall developmental mortality, sex ratio, fertilization rate, diploid male survival rate, and total eggs laid per female *H. hebetor* for matched and unmatched matings at 20° and 27° C. The average number of eggs laid ranged between 105 and 135 per female with significantly fewer eggs laid by females from unmatched matings at 27°C (interaction between temperature and mating type: $P < 0.05$). The egg hatch rate was not significantly affected by temperature, but was significantly lower when eggs were laid by females of a matched mating ($P < 0.001$). Similarly, developmental mortality was significantly higher in the matched matings ($P < 0.001$) but not significantly

affected by temperature. The estimated fertilization rates were not significantly affected by temperature or mating type, but sex ratios were significantly more male-biased in the matched matings ($P < 0.01$), as expected when most diploid males die during development. Finally, the estimated diploid male survival rate was significantly higher at 20° C than at 27° C ($P = 0.05$).

DISCUSSION

Our results are not consistent with Butcher's finding of a lack of CSD when development occurs at low temperatures (Butcher 1998, Butcher et al. 1996, 1998). Instead, our findings suggest that the developmental temperature has no effect on sex determination mode. What could account for this difference? One possibility is that our negative results are an artifact of our *H. hebetor* being *Wolbachia*-free. We have recently screened two wild populations of *H. hebetor* for *Wolbachia* and found both to be infected (M.F.A., L.A.W., G.E.H. unpublished). It is therefore possible that the stocks of *H. hebetor* used by Whiting & Anderson (1932) and Butcher were infected with *Wolbachia.* If temperature-dependent sex determination is only expressed by *Wolbachia*-infected *H. hebetor,* then our lack of a temperature effect could be attributed to the absence of *Wolbachia.* However, a separate study in which temperature effects on CSD were evaluated in *Wolbachia*-infected *H. hebetor* yielded results similar to the ones reported here (Weiser et al., in preparation).

Our failure to find temperature-dependent CSD parallels Beukeboom's (2001) failure to find temperature effects on CSD in a sexual strain of *V. canescens,* which had originally been reported by Butcher et al. (1998). In both of these cases, the potential for intraspecific variation in sex determination and related traits cannot be excluded. It is becoming clear that sex determination mode is evolutionarily flexible and that relatively closely related taxa can have different forms of sex determination (Werren & Beukeboom 1998, Kraak & Pen 2002, Cook 2002). In some animals, variation in the sex determination mode has even been found among populations of the same species. Examples of non-symbiont-related intraspecific variation in sex determination include a fish, the woodlouse *Armadillidium vulgare,* a shrimp and the housefly, *Musca domestica* (reviewed by Werren & Beukeboom 1998 and Cook 2002). Sex determination has not been reported to differ within species in Hymenoptera, but it is clear that some species of braconids have sl-CSD while other species do not (Beukeboom et al. 2000, Wu et al. 2003) and this level of variation appears to be present within a single braconid genus as well. CSD has been identified in *Cotesia rubecula* (Stouthamer et al. 1992 and personal communication of W.W.M. Steiner to L.A.W.), but not *C. flavipes* (Niyibigira 2003). Determining whether there is variation among hymenopteran strains in sex determination mode or diploid male survivorship will have to await side-by-side studies of strains that are reported to be temperature-sensitive and temperature-insensitive.

Although the reasons for the difference between our results and those of Butcher (1998) remain unclear, it is still conceivable that the temperature that females experience prior to oviposition has an effect on sex determination. Whiting and Anderson (1932) reported a lower incidence of diploid males from females that were both held and allowed to oviposit at 20°C rather than 30°C. Because Whiting and Anderson did not report data on fecundity, egg hatch, or developmental survivorship, it is not clear whether these differences are due to true differences in sex determination (i.e., sex allele homozygotes developing as females) or to differences in the survivorship of diploid males. For this reason, and because Butcher (1998) had identified the egg or early larva as the critical stage for sex determination, we did

not test for an effect of the temperature experienced by females on sex determination. Thus, our experiment is not a repeat of Whiting and Anderson's (1932) work and leaves open the possibility that the temperature female *H. hebetor* experience prior to oviposition affects sex determination.

While all species of *Habrobracon* studied to date have exhibited sl-CSD, the survivorship of diploid males varies greatly among species (Speicher & Speicher 1940, Clark et al. 1963, Holloway et al. 1999) and within *H. hebetor* (Whiting & Anderson 1932; Petters & Mettus 1980; this study). In our study, diploid male survivorship was significantly higher at the low temperature. Temperature-dependent survivorship rates of diploid males can have important implications for the population-level effects of CSD in the field. Viable diploid males that are sterile yet that participate in mating have stronger (negative) effects on population sizes than inviable diploid males, because females mated to these sterile diploid males are constrained to producing only haploid sons (Stouthamer et al. 1992; Holloway et al. 1999). Our finding suggests that these dynamics may be linked to temperature fluctuations in the field.

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